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Use of Miracil D to Suppress Bacterial Ribonucleic Acid and Protein Synthesis During Bacteriophage MS2 Infection

JANE HARRIS CRAMER[1] AND ROBERT L. SINSHEIMER
Biology Division, California Institute of Technology, Pasadena, California 91109

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Under certain culture conditions, Miracil (35 μg/ml) halts the growth of uninfected *Escherichia coli*. Cellular ribonucleic acid (RNA) synthesis is almost completely suppressed, whereas deoxyribonucleic acid and protein synthesis are inhibited to a lesser extent. When the drug is added to host bacteria prior to infection with bacteriophage MS2, the phage adsorb to the cells, but penetration of the viral RNA is inhibited. Penetration may be achieved without further viral development by infection in the presence of chloramphenicol. If the bacteria are infected with MS2 in the presence of chloramphenicol, subsequently washed to remove the chloramphenicol, and then treated with Miracil at any time between 0 and 20 min postinfection, a second viral function is inhibited and the yield of progeny phage is reduced. Addition of the drug after 20 min postinfection does not inhibit the infection process. When Miracil is present from early times in infection, only a limited synthesis of both double- and single-stranded virus-specific RNA is observed. The viral RNA species thus produced do not appear to differ from those made in the absence of the drug. A comparison of the activities of the viral RNA synthetase produced during the course of infection in the presence and in the absence of Miracil suggests that a possible cause of the inhibition is the synthesis of an unstable enzyme in the presence of the drug.

During infection of *Escherichia coli* with most ribonucleic acid (RNA) bacteriophages, a high rate of normal cellular macromolecular synthesis persists and it is difficult to label phage-specific RNA and protein species selectively with radioisotopes. Actinomycin, rifampin, and ultraviolet irradiation of the bacterial cells have been used as methods to inhibit host RNA synthesis. However, viral replication may be abnormal after any of these treatments since phage yield is markedly reduced, and some RNA species not found in untreated infected cells are observed (5, 7, 9, 14).

This report describes a new method, using the drug Miracil D, for suppressing *E. coli*-directed RNA and protein synthesis during infection by the RNA bacteriophage MS2. Miracil acts in a manner similar to actinomycin, inhibiting deoxyribonucleic acid (DNA)-dependent RNA synthesis by complexing with the DNA (21). In contrast to actinomycin, however, the host cells are permeable to Miracil and need not be sensitized to drug action by preliminary treatment with tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) (11). This not only simplifies experimental manipulations but eliminates artifacts resulting from the Tris-EDTA treatment, which is known to affect cellular metabolism extensively under certain conditions (12, 15). The experiments discussed here deal with selective labeling of viral RNA. Evidence for selective labeling of viral proteins in the presence of Miracil has been presented elsewhere (3).

MATERIALS AND METHODS

Chemicals. Thymidine-*methyl*-^3H, uracil-6-^3H, and *l*-leucine-4,5-^3H were purchased from Schwartz BioResearch, Inc. ^{32}P, carrier-free, was obtained from Mallinckrodt Nuclear. Chloramphenicol was a gift from Parke, Davis & Co. Miracil D (lucanthone HCl) was the gift of Burroughs Wellcome and Co. through the courtesy of George Hitchings. Miracil stock solutions at 1 mg/ml were made up fresh in distilled water every 1 or 2 days because the drug loses its effectiveness when kept in solution over longer periods of time.

[1] Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison 53706.

Media. MS broth was the same as that described by Pfeifer et al. (17), except the $CaCl_2$ concentration was reduced to 10^{-4} M. The *p*H of this medium is 6.8.

TPG-minimal contained NaCl 0.5 g; KCl, 8.0 g; NH_4Cl, 1.1 g; Trizma base, 12.1 g; KH_2PO_4, 0.8 g; sodium pyruvate, 0.8 g; 1.0 ml of 20% $MgCl_2 \cdot 6H_2O$; and 1.0 ml of 0.16 M Na_2SO_4 per liter of distilled water. The *p*H was adjusted to 7.4. After autoclaving, 20 ml of 10% glucose and 1.0 ml of 0.1 M $CaCl_2$ were added.

TPG-c was TPG-minimal plus 3.0 g of vitamin-free Casamino Acids (Difco) per liter.

TPA (low phosphate) was TPG-minimal with the KH_2PO_4 concentration reduced to 0.046 g/liter and the addition of 2.7 g of amino acid mixture per liter (special 20 natural *l*-amino acid mixture, Nutritional Biochemicals Corporation).

Although the $CaCl_2$ concentration in all these media was reduced to 10^{-4} M, phage production and the extent and kinetics of infective center formation were the same as in the higher Ca^{2+} concentration (2×10^{-3} M) usually used for RNA phage infection.

Biological assays. All dilutions of phage and cells were made through MS broth from which the glucose, $CaCl_2$, and thiamine had been omitted. Plaque assays and viable counts were done by using the agar layer technique. *E. coli* C3000 grown to 10^8 to 2×10^8 cells/ml in MS broth was used as plating bacteria.

Infective centers were defined as infected cell complexes and did not include free phage particles. They were measured by adding 0.1 ml of an infected culture to 0.9 ml of a 1:300 dilution of MS2 antiserum ($K = 1{,}040$) at 4 C. After 5 min in antiserum, the infected cells were diluted and plated immediately.

To titer intracellular plus extracellular phage, 0.1 ml of an infected culture was diluted into 0.8 ml of 0.033 M Tris, *p*H 8. Then 0.1 ml of lysozyme (4 mg/ml in 0.25 M Tris, *p*H 8.1) and 0.2 ml of EDTA (4% in distilled water) were added and the samples were incubated for 10 min at 0 C. The cells were lysed by the addition of 0.8 ml of 0.1% sodium dodecyl sulfate (SDS; Matheson, Coleman, and Bell), and the phage were titered.

Infectivity assays of MS2 RNA were performed with the *E. coli* spheroplast system of Strauss and Sinsheimer (18).

Chloramphenicol synchronization of infection. The synchronization procedure is an adaptation of that used by Kelly et al. (10). Uninfected cells were incubated for 5 min in chloramphenicol, 25 μg/ml, and the culture was infected with the desired multiplicity of MS2. After an additional 5 min of incubation, the cells were collected by filtration on a cellulose nitrate filter (Millipore HAWP, 0.45 μm pore size), washed with two volumes of media to remove the chloramphenicol, and resuspended in growth medium warmed to 37 C. This was taken as time zero. The phage RNA penetrates in the presence of chloramphenicol, but the infection proceeds no further until the drug is removed since the viral RNA synthetase is not synthesized in its presence.

Radioactivity measurements. Incorporation of ^{3}H-uracil and ^{3}H-leucine into cold and hot trichloroacetic acid-insoluble material, respectively, was measured by precipitating 0.5-ml samples of labeled cultures with 2.0 ml of 7% trichloroacetic acid. Incorporation of ^{3}H-thymidine into cold acid-insoluble material was also measured by precipitation of labeled samples with 7% trichloroacetic acid, except that the samples were first incubated in 0.5 N KOH at 37 C for 12 hr. Denatured calf thymus DNA (100 μg) was added as carrier to all radioactive samples.

Labeled RNA was detected on sucrose gradients by the following method. Each gradient fraction was diluted with 1.0 ml of a buffer containing 0.1 M Tris (*p*H 7.4), 0.01 M $MgCl_2$, and 0.1 M NaCl (TMS buffer). Carrier was added, and the samples were precipitated with cold trichloroacetic acid as above. If the ribonuclease resistance of the RNA in each fraction was to be determined, one half of the sample was precipitated and counted; the other half was incubated at 37 C with 25 μg of ribonuclease per ml (Bovine Pancreas, Type I-A, Sigma Chemical Co.) for 30 min to digest any single-stranded RNA prior to precipitation and counting.

Acid precipitates were collected on Whatman glass filters. Dried filters were placed in scintillation fluid which contained 42 ml of Liquifluor (New England Nuclear Corp.) per liter of toluene and counted in a Beckman scintillation counter.

Phenol extraction and sedimentation of RNA. All RNA extraction procedures were carried out at 0 to 4 C. Cells were chilled, collected by centrifugation, and resuspended in 1.0 ml of 0.1 M Tris, *p*H 7.4. After the addition of 0.2 ml of 10% SDS, the samples were vortexed vigorously and frozen and thawed once. This treatment was sufficient to lyse the cells.

The lysate was extracted twice with equal volumes of water-saturated phenol and once with ether, and the RNA was precipitated by the addition of sodium acetate to 0.3 M and three volumes of 100% ethanol. After 8 to 10 hr at −20 C, the precipitate was collected by centrifugation and resuspended either in TMS buffer, if the sample was to be assayed for ribonuclease-resistant RNA, or in 0.1 M Tris, *p*H 7.4, if the RNA was to be analyzed by sucrose gradient sedimentation.

Five milliliters of 5 to 20% (w/v) linear sucrose gradients in 0.1 M Tris, *p*H 7.4, were poured at room temperature and chilled to 4 C. RNA samples (0.1 ml) in the same buffer were layered on the gradients. The length and speed of centrifugation are designated in the figure legends for each experiment. All sucrose gradients were collected dropwise from the bottom of the centrifuge tube.

Preparation of ^{32}P-labeled MS2. ^{32}P-labeled MS2 virus was prepared by the method of Godson and Sinsheimer (8).

Viral RNA synthetase assay. Cells which were to be assayed for viral RNA synthetase were disrupted by the Brij lysis method described by Godson and Sinsheimer (8), and the crude lysate was used as the enzyme source. Since the lysate contains 50 μg of deoxyribonuclease per ml, it is highly unlikely that

any activity in the assay is due to DNA-dependent RNA polymerase. Protein concentration was determined by the Folin procedure (13).

The reaction mixture contained in 0.25 ml: Tris (*p*H 7.4), 20 μmoles; $MgSO_4$, 3 μmoles; adenosine triphosphate, uridine triphosphate, and guanosine triphosphate, 0.2 μmole each; cytidine triphosphate (CTP), 0.05 μmole; ^{3}H-CTP, 0.5 μCi, 4×10^{-4} μmole (Schwartz BioResearch, Inc.). After incubation at 37 C for 6 min, the reaction was stopped by the addition of 2.0 ml of 7% trichloroacetic acid, carrier was added, and the precipitate was collected on glass filters. Under these conditions, incorporation of ^{3}H-CTP into acid-precipitable material was linear with time for approximately 8 to 10 min. Negligible incorporation of ^{3}H-CTP was observed when crude lysates of uninfected cells were assayed.

RESULTS

Growth and metabolism of uninfected bacteria in Miracil. The effect of Miracil D on the growth and metabolism of *E. coli* varies from strain to strain as well as in the same bacterial strain grown in different media (20). Under different culture conditions, the same drug concentration may lyse the cells, halt cell growth without causing lysis, or have no apparent effect on the cells at all. Before examining the effect of Miracil on the MS2 infection process, we tested its action on *E. coli* MRE 600, a ribonuclease I^- strain (2), the strain to be used as host in subsequent MS2 experiments. Two differently supplemented minimal media routinely used for MS2 growth were used. In TPG-c with 15 μg of Miracil/ml, bacterial growth continues but is markedly inhibited. In the same medium with 25 μg/ml, the cells cease to grow after one generation, whereas in 35 μg/ml, no growth occurs after addition of the drug. In the second medium, TPA, Miracil at the same concentrations has very little effect on cell growth. When cultures grown in a glucose-salts minimal medium, TPG-minimal, or a complex medium, MS broth, were treated with Miracil, the results were the same as those observed in TPG-c. In both cases 35 μg/ml halted cell growth without causing lysis.

In each of these experiments, the *p*H of the medium was as described above. Similar results were obtained when the *p*H of each medium was varied from 6.5 to 7.8. Since small differences in *p*H might affect the solubility and hence the activity of Miracil, the *p*H of each medium was checked again immediately prior to addition of the drug to be certain no change had occurred during growth of the cells. We had found that the sensitivity of the cells to Miracil was inhibited by high Ca^{2+} concentrations (2×10^{-3} M); therefore all media contained only 10^{-4} M Ca^{2+} (*see above*).

In the presence of Miracil, incorporation of ^{3}H-thymidine into DNA in cultures grown in TPG-c is normal for the first 5 min and then continues at a reduced rate (Fig. 1a). At 35 μg/ml, the rate of incorporation decreases steadily for about one generation time, and after 40 min virtually no uptake occurs. Total synthesis in 40 min is approximately 10% of that in the untreated culture.

The inhibition of RNA and protein synthesis is more rapid (Fig. 1b, c). The effect on both uracil and leucine uptake is evident after 5 min. By this time, total ^{3}H-uracil incorporation in 35 μg of Miracil/ml is only 12% of that in the untreated culture. (Experiments not shown have demonstrated that, after only 2 min in Miracil at 35 μg/ml, the final suppressed rate of RNA synthesis, about 5% of that in an untreated culture, is achieved.) Incorporation of ^{3}H-leucine is also reduced, but at 35 μg/ml it is not as depressed as ^{3}H-uracil incorporation.

Miracil at 15 and 25 μg/ml also inhibits bacterial macromolecular synthesis but to a lesser extent than at 35 μg/ml. The degree of inhibition with a given Miracil concentration is quite reproducible from one experiment to another.

Effect of Miracil on the MS2 infection process. For Miracil to be useful in studies of MS2 infection, it is necessary to use drug doses which reduce host RNA and protein synthesis to low levels but which do not severely disrupt the infection process. Figure 2 shows the effect of increasing Miracil concentrations on infective center formation and progeny phage yield when the drug is added 1 min after the addition of phage. Infective centers are slightly reduced at lower concentrations but drop off sharply between 30 and 40 μg/ml. This effect at higher concentrations has been observed repeatedly. The effect on phage yield is even more pronounced than the decline in the number of infective centers. The average burst size per infective center decreases exponentially with increasing drug concentration above 5 μg/ml.

We first studied the effect of Miracil on the early steps of infection-adsorption and penetration. RNA phage will adsorb to nonmetabolizing cells, either at 0 C or in the presence of metabolic inhibitors, but for the RNA to penetrate, cellular metabolic activity is required (4). An *E. coli* culture was divided into two equal portions, and one half was treated with Miracil, 40 μg/ml, for 3 min. Both cultures were chilled rapidly to 0 C, ^{32}P-labeled MS2 was added to each at a multiplicity of one, and at various times samples

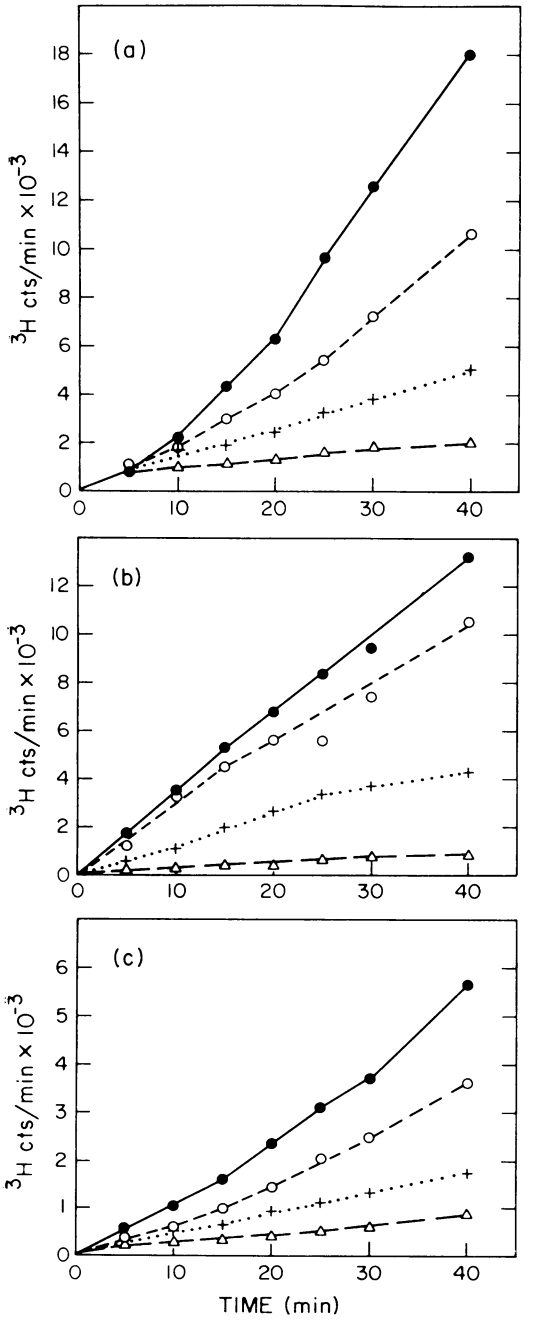

FIG. 1. *Inhibition of bacterial DNA (a), RNA (b), and protein (c) synthesis by Miracil. A culture of E. coli MRE 600 was grown to 2 × 10^8 cells/ml in TPG-c (containing 4 μg of thymidine/ml and 5 μg of uracil/ml) and split into three portions. ^{3}H-thymidine was added to the first to a concentration of 1 μCi/ml, ^{3}H-uracil was added to the second to a concentration of 0.5 μCi/ml, and ^{3}H-leucine was added to the third to a concentration of 1.0 μCi/ml. Each of these subcultures was divided immediately into four equal portions containing no Miracil (●), 15 μg of Miracil/ml, (○), 25 μg of Miracil/ml, (+), and 35 μg of Miracil/ml, (△).*

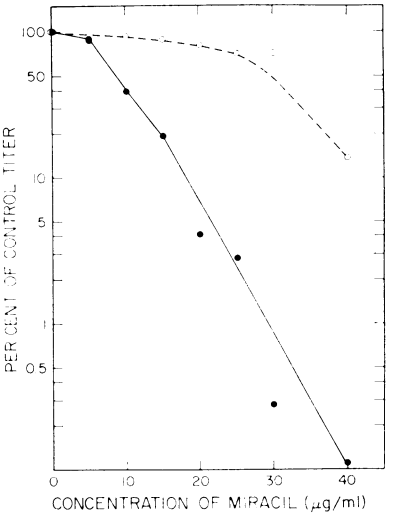

FIG. 2. *Inhibition of RNA phage growth by increasing concentrations of Miracil. E. coli cells were grown to 2 × 10^8/ml in TPG-c and infected with MS2 at a multiplicity of 5. The culture was divided into eight equal portions, and Miracil, at the indicated concentrations, was added 1 min after infection. At 6 min postinfection, infective centers were measured, and, after incubating the cultures in Miracil for 70 min, intra- plus extracellular phage were titered. The values are expressed as per cent of the control culture which received no Miracil. ○, Infective centers; ●, intra- plus extracellular phage.*

were removed, and the cells were pelleted and counted. The amount of radioactivity in the pellets from the treated and untreated cultures was the same, indicating that the amount of phage adsorption was equivalent in the two cultures. If the cells were blended prior to pelleting to remove the pili, and thus the adsorbed phage, almost no radioactivity was found in the cell pellet. To assay for penetration, we chilled Miracil-treated and untreated cultures, as in the preceding experiment, and allowed ^{32}P-labeled MS2 to adsorb at 0 C for 10 min. The cultures were then warmed rapidly to 37 C, samples were removed at various times and blended to remove adsorbed but uninjected phage, and the cells were pelleted and counted. By 10 min postinfection, only 10% as much radioactive RNA had penetrated the Miracil-

treated cells as the untreated cells. The same results were obtained if the phage were not preadsorbed at 0 C. These results indicate that MS2 adsorbs in the presence of Miracil, but only a small proportion of the viral RNA penetrates. Apparently, some metabolic activity required for penetration is disrupted by Miracil.

Viral RNA penetration is asynchronous and is not complete until 8 or 9 min postinfection (R. B. Kelly, Ph.D. thesis, California Institute of Technology, Pasadena, 1967). So that penetration could be completed and the effects of Miracil on subsequent steps of infection examined, bacteria were infected synchronously in chloramphenicol (*see above*), and Miracil was added subsequent to the removal of the chloramphenicol. Figure 3 shows the inhibitory effect of Miracil on phage yield in chloramphenicol-synchronized cells when the drug is added at succeeding times during infection. Maximum inhibition occurs when Miracil is added during the first few minutes of infection. The infection becomes progressively less sensitive to the drug, and, after 20 min, phage yield is unaffected by the presence of Miracil. Progeny phage are just beginning to appear at this time; therefore, viral maturation and assembly apparently are unaffected by the drug. In addition, most of the coat and maturation protein necessary for phage production is synthesized later than 20 min of infection (19); therefore, viral protein synthesis is probably also insensitive to Miracil action. Whatever function is inhibited by the drug is either completed or no longer essential for phage production by this time.

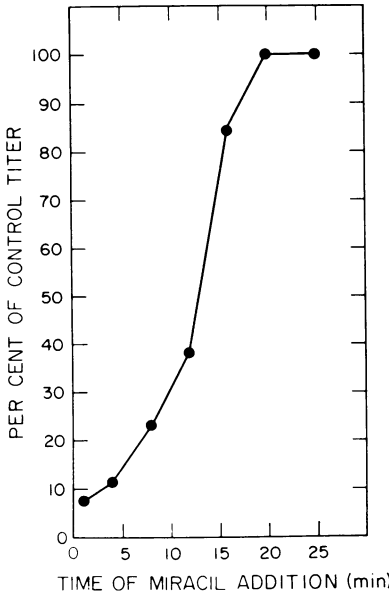

FIG. 3. *Time course of Miracil inhibition. E. coli cells were grown to 2 × 10⁸/ml in TPG-c. Chloramphenicol was added to synchronize the infection as described in the text, and the culture was infected with MS2 at a multiplicity of 10. Five minutes after the addition of MS2, the chloramphenicol was removed and the cells were resuspended in TPG-c warmed to 37 C. This was taken as time zero. At the indicated times, Miracil, 35 μg/ml, was added to samples of the culture. All samples were incubated to 70 min postinfection, and the intra- plus extracellular phage were titered. Phage yield is expressed as per cent of the titer at 70 min in a culture which had not been treated with Miracil.*

The inhibitory effect on phage yield which is observed when Miracil is added early in infection is partially reversible. A culture was infected in the presence of chloramphenicol, washed, and resuspended. One minute later, Miracil (35 μg/ml) was added (exactly as for the first time point in the preceding experiment), and at short intervals up to 20 min after addition of Miracil, samples of the culture were diluted 10,000-fold to reduce the drug concentration, incubated for 2 hr, and titered. In the sample which was diluted after exposure to Miracil for 1 min, the number of progeny phage produced was normal. This short incubation in Miracil is not completely without effect, however, since the lag period before the appearance of progeny phage is longer than in the control culture which was diluted just prior to the addition of Miracil. As incubation times in Miracil are increased to longer than 1 min, the phage yield begins to decrease; however, even after the infection has proceeded for 20 min in the presence of Miracil, partial recovery is observed since the phage titer in the culture which was diluted out of Miracil after 20 min was five times that of a culture which remained in Miracil during the entire 2-hr incubation period.

In the experiment above, the decreased phage yield after exposure of infected cells to Miracil for 10 min or less is due to a drop in the number of infected cells, whereas the average burst size per infective center remains constant. After longer incubation periods in Miracil, the average burst size also begins to decline.

A parallel experiment was done to determine whether the effect of Miracil on the colony-forming ability of uninfected cells was also reversible. Cells were incubated for the same intervals in Miracil at 35 μg/ml and then were diluted and plated immediately. There is a striking similarity between the proportion of cells which are still able to form colonies and the extent to which infection can proceed. This relationship may simply be fortuitous. However,

it may be an indication that only those cells which can still divide and form colonies after treatment with Miracil are able to provide some function which is required for MS2 infection.

Phage RNA synthesis. Synthesis of virus-specific RNA in Miracil-treated culture was studied in an attempt to determine what step in the replication cycle is blocked by the drug once penetration is complete, and to see whether the virus-specific RNA species which continue to be synthesized are altered in the presence of the drug. Miracil was added at 3 min post-infection to one half of a culture infected with ^{32}P-labeled MS2, and the fate of the infecting viral RNA was followed to see if parental RF was formed. Samples from the culture with and without Miracil were removed at 3-min intervals until 15 min postinfection, and the per cent of parental RNA in ribonuclease-resistant form was determined in each. Although the infection was not synchronized in this experiment, only the fate of the RNA which has penetrated the cells is observed since the culture was blended to remove uneclipsed phage particles prior to RNA extraction. In the presence of the drug, conversion of labeled RNA to ribonuclease-resistant form ceases after a few minutes, but that in the untreated culture continues. By 15 min post-infection, the total percentage of ^{32}P-labeled ribonuclease-resistant material formed in the Miracil-treated culture is less than half of that in the infected culture with no Miracil.

To see whether alteration or degradation of the infecting RNA strand in the presence of Miracil might be responsible for the decreased parental RF formation, the RNA from the treated and untreated 12-min samples in the above experiment was analyzed by sucrose gradient sedimentation (Fig. 4). The RNA from the culture without Miracil contains about twice as much ribonuclease-resistant material sedimenting in the viral replicative intermediate region from 16 to 25*S* as the Miracil-treated culture, but in all other respects the sedimentation patterns of the two RNA samples are very similar. Breakdown of viral RNA into slower sedimenting species and possibly limited incorporation of ^{32}P into host RNA have occurred in both cultures; however, degradation of parental RNA is not unusual this late in infection, and the viral RNA from the Miracil-treated culture is not more extensively degraded than that from the untreated culture. If the drug causes minor alterations in the infecting RNA or binds to the RNA to interfere with its conversion to RF, the sedimentation properties of the parental RNA would not be expected to change markedly.

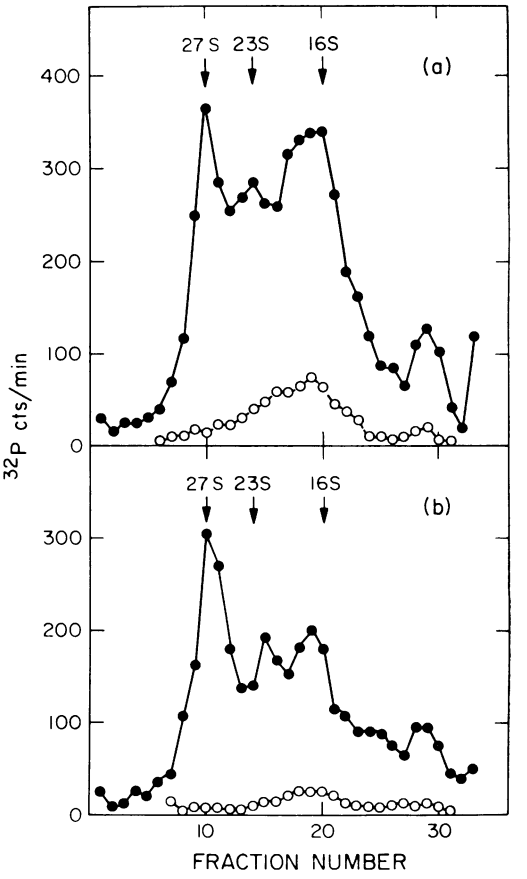

FIG. 4. *Sedimentation pattern of ^{32}P-labeled viral RNA isolated from Miracil-treated cells. The RNA samples from the 12-min time point in the experiment discussed in the text were sedimented on 5-ml 5 to 20% sucrose gradients; a, untreated; b, Miracil-treated. Centrifugation was at 180,000 × g for 4 hr in an SW65 rotor at 4 C. Each gradient fraction was split; one half was precipitated directly and the other half was assayed for ribonuclease resistance before precipitation and counting. (The direction of sedimentation was from right to left.)* ●, *Total ^{32}P counts per minute;* ○, *ribonuclease-resistant ^{32}P counts per minute.*

The next experiment was designed to determine whether synthesis of both viral and complementary strands, represented by infective and ribonuclease-resistant RNA, respectively, could continue in the presence of Miracil. Samples were removed from an ^{3}H-uracil-labeled, infected culture at intervals during infection. One half was assayed for infective RNA and ribonuclease-resistant material; the other half was treated with Miracil, incubated to 40 min postinfection, and then assayed for infectivity

and ribonuclease resistance to see whether the number of viral and complementary strands had increased in the presence of the drug. Table 1 shows the results for infective RNA. Clearly, viral strand synthesis continues in Miracil. When the drug is added early in infection, only a limited increase is seen, but between 20 and 25 min postinfection, RNA synthesis escapes from the inhibitory effect and a normal amount of infective RNA is made. Table 2 shows that the amount of ribonuclease-resistant RNA also increases after the addition of Miracil; thus, complementary strand synthesis must also continue in the presence of the drug.

To see whether any of the virus-specific RNA species synthesized in the presence of Miracil differed from those found in its absence, the following experiment was done. An infected culture was divided at 8 min postinfection, and one half was treated with Miracil, 35 μg/ml. (Addition of Miracil at 8 min in this and the following experiment allowed penetration to be completed.) Both samples were labeled with ^{3}H-uracil from 10 to 30 min postinfection. An uninfected culture was Miracil-treated and labeled at the same time to determine which bacterial RNA species were being synthesized under these conditions. Figure 5 shows the sedimentation pattern of the RNA from each culture. Host RNA synthesis at this Miracil concentration is only 5 to 10% of the rate in untreated cultures. Analysis of the RNA from the uninfected culture shows that 23, 16, and 4*S* all are produced in reduced amounts in the presence of the drug.

In the infected sample without Miracil, the 27*S* viral peak is distinguishable, and the position of replicative intermediate molecules in the 16 to 25*S* region can be detected by their partial resistance to ribonuclease but any other information about viral RNA species is obscured by the large amount of radioactivity in host RNA. In Miracil, virus-specific RNA species are readily distinguishable. Although the amount of 27*S* and double-stranded RNA is reduced compared to the untreated sample since Miracil was added so early in infection, the phage-specific RNA synthesized in the presence of the drug does not differ from that produced in its absence, at least with regard to sedimentation properties. (Due to the difference in scale in Fig. 5b and c, there appears to be no significant increase in the amount of ribonuclease-resistant material in the infected culture compared with the unin-

TABLE 1. *Effect of Miracil on synthesis of infective viral RNA*[a]

t[b]	Relative infectivity at t[c] (%)	Relative infectivity at 40 min with Miracil present from t to 40 min[c] (%)
4	0.02	2.3
8	0.07	0.54
12	0.43	11.0
16	3.3	13.1
20	8.3	17.4
25	25.8	98.0
40	100	100

[a] *E. coli* cells were grown to 2×10^8/ml in TPG-c plus uracil, 5 μg/ml, and infected with MS2 at a multiplicity of 5. ^{3}H-uracil was added to a concentration of 0.5 μCi/ml. At the indicated times, two samples were removed; one was chilled immediately, and the RNA was extracted with phenol. The other was treated with Miracil, 35 μg/ml, and incubated at 37 C until 40 min postinfection, when all the cultures were chilled, and the RNA from each was extracted with phenol. Each sample was assayed for infective viral RNA.

[b] Time of sampling and of Miracil addition in minutes postinfection.

[c] The amount of infective RNA in the samples taken at each time point is expressed as a percentage of the amount at 40 min in a culture not treated with Miracil (relative infectivity at t). The amount of infective RNA at 40 min in the cultures treated with Miracil from t to 40 min is also expressed as a percentage of the amount in the 40-min untreated culture (relative infectivity at 40 min with Miracil present from t to 40 min).

TABLE 2. *Effect of Miracil on synthesis of ribonuclease-resistant RNA*[a]

t[b]	Relative ribonuclease resistance at t (%)	Relative ribonuclease resistance at 40 min with Miracil present from t to 40 min (%)
4	9.1	27.0
8	21.4	48.6
12	32.0	53.5
16	46.0	58.2
20	69.8	78.3
25	85.0	87.0
40	100	100

[a] The samples from the experiment described in the legend to Table 1 were also treated with ribonuclease to determine the amount of radioactivity which had been incorporated into ribonuclease-resistant material. The number of ribonuclease-resistant counts in each sample is expressed as a percentage (relative ribonuclease resistance) of the number in a culture at 40 min which had not been treated with Miracil.

[b] Time of sampling and of Miracil addition in minutes postinfection.

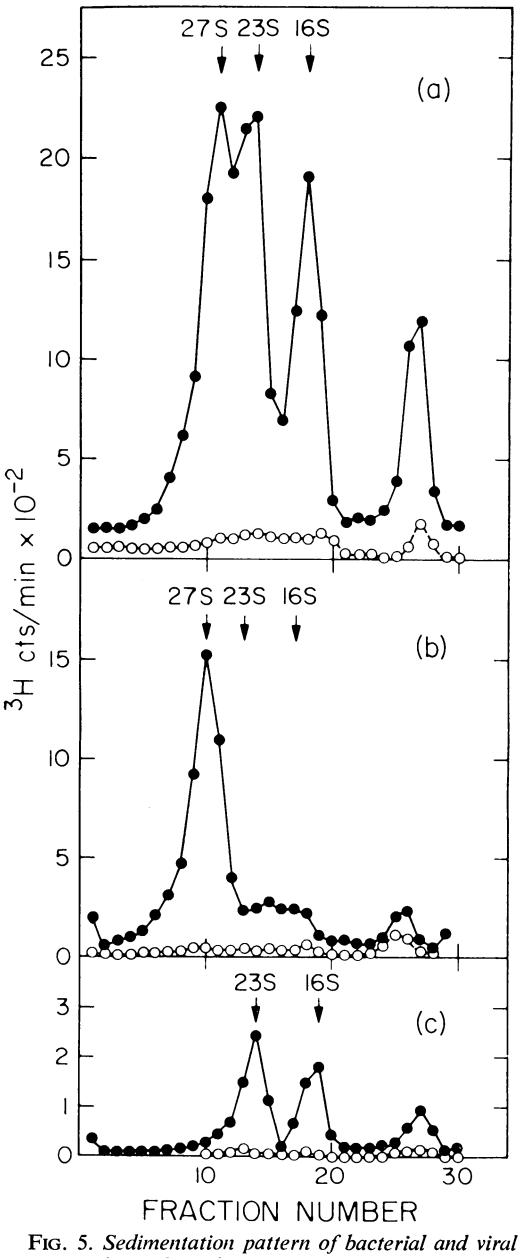

FIG. 5. *Sedimentation pattern of bacterial and viral RNA synthesized in the presence of Miracil. E. coli cells were grown to 2 × 10^8/ml in TPG-c plus uracil, 5 μg/ml. A sample was removed for the uninfected culture, and the remainder was infected with MS2 at a multiplicity of 3. At 8 min postinfection, the infected portion was split; Miracil, 35 μg/ml, was added to one half and to the uninfected culture. Two minutes later, ^{3}H-uracil was added to all three cultures to a concentration of 0.5 μCi/ml. The cells were incubated to 30 min postinfection and harvested, and the RNA was extracted with phenol. Equal portions of the three RNA preparations were analyzed by sucrose gradient sedimentation. Centrifugation was at 300,000 × g for 2 hr in an SW65 rotor at 4 C. One half of each gradient fraction was precipitated directly. The other half was treated with ribonuclease before precipitation and counting. ●, Total ^{3}H counts per minute; ○, ribonuclease-resistant ^{3}H counts per minute. a, Infected culture, no Miracil; b, infected culture in Miracil, 35 μg/ml; c, uninfected culture in Miracil, 35 μg/ml (note change of scale).*

fected one. The actual amount of ribonuclease-resistant radioactivity, however, is five times higher in the infected Miracil-treated culture.) There is still some residual incorporation into host 4*S* RNA and probably also into ribosomal RNA, but the proportion of labeled bacterial RNA is far less than in the absence of the drug.

Phage RNA synthetase production. The results above indicate that both viral and complementary strand synthesis can continue in Miracil-treated, infected cells, but only to a limited extent when the drug is added before 20 min of infection, and that the viral RNA species synthesized in the presence of the drug appear normal. These results would be explained if Miracil inhibited viral RNA synthetase activity. The strong similarity between the time course of Miracil inhibition (Fig. 3) and the time course of RNA synthetase production (Fig. 6) also suggests that Miracil might be affecting the enzyme activity in some way.

When a crude lysate was assayed for replicase activity at 20 min postinfection, Miracil did not inhibit triphosphate incorporation even at concentrations as high as 200 μg/ml. The conditions of the assay were as described above. Miracil is soluble at the *p*H of the assay (7.2 to 7.4), and, although the Mg^{2+} concentration is approximately 10^{-3} M, it is unlikely that the presence of this cation in the assay is preventing the inhibitory activity of Miracil since DNA-dependent RNA polymerase activity was 98% inhibited by less than 20 μg of Miracil per ml in the presence of similar Mg^{2+} concentrations (20). These results suggest that the drug does not halt RNA synthesis by interacting directly with either the enzyme or its template. However, since the source of enzyme and template was a whole cell lysate, it is possible that the incorporation observed in vitro merely reflects the completion of viral and complementary strands initiated in vivo and does not include any reinitiation of synthesis. Thus, although Miracil appears unable to affect the enzyme or template once synthesis has begun, we cannot determine for certain with this type of assay whether Miracil interferes with

enzyme-template binding or with initiation of synthesis.

Infected cultures with and without Miracil were assayed for synthetase activity at intervals during infection to test the possibility that the drug prevents formation of active enzyme rather than inhibits its activity (Fig. 6). Very little enzyme activity was present when Miracil was added, but by 20 min postinfection the level of activity in the Miracil-treated culture was almost equal to that in the untreated culture. What is striking is that the RNA synthetase activity in the preparation from the Miracil-treated culture drops off sharply after 20 min, whereas that in the control without Miracil remains constant. This is not due to selective lysis of infected cells in the Miracil-treated culture and loss of the synthetase into the supernatant fluid, since the cell count remained constant. Thus it seems possible that the presence of Miracil during production of the RNA synthetase may cause some alteration in the activity of the enzyme.

DISCUSSION

Under certain growth conditions, Miracil D at 35 to 40 μg/ml almost completely inhibits RNA synthesis in intact *E. coli* MRE 600 cells. Suppression of DNA and protein synthesis is not as great. Similar results were obtained by Weinstein et al. (20) in a different *E. coli* strain. In vitro the drug complexes with DNA (21) to inhibit *E. coli* RNA polymerase activity and, to a much lesser extent, DNA polymerase activity. Miracil also binds slightly to RNA, but, since it does not inhibit amino acid incorporation in vitro with either natural or synthetic messenger (20), its inhibitory effect on protein synthesis in vivo is probably secondary, resulting from depletion of cellular messenger RNA.

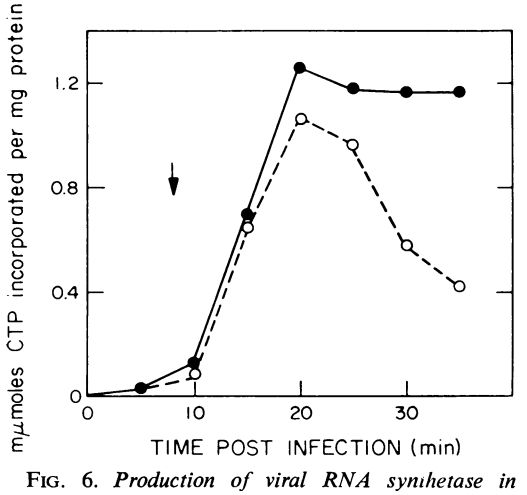

FIG. 6. *Production of viral RNA synthetase in Miracil. E. coli cells were grown to 4 × 10⁸/ml in TPG-c and infected with MS2 at a multiplicity of 2. At 8 min postinfection, the culture was divided and Miracil, 40 μg/ml, was added to one half. At various times, samples were removed from each culture, chilled rapidly, and lysed. Each sample was assayed for viral RNA synthetase as described in the text. The arrow indicates the time at which Miracil was added. ●, Micromoles of cytosine triphosphate incorporated per milligram of protein in crude enzyme preparations from the culture without Miracil; ○, from the culture with Miracil.*

The inhibitory effect of Miracil varies widely when cells are grown in different media. The effect is unrelated to bacterial growth rate since the drug works equally well in minimal and in complex media. Nor are *p*H differences (in the range 6.5 to 7.8) responsible for the variation. The sensitivity of cells to Miracil is, however, at least partly dependent on the ionic composition of the medium. It is interesting, with regard to our observations on the effect of Ca^{2+}, that the inhibitory effects of Miracil D are also prevented by polyamines (20). The concentrations of divalent cations, phosphates, and possibly other salts as well appear to play a complex role in determining cellular susceptibility to Miracil probably by altering the permeability characteristics of the cells.

Miracil appears to inhibit the MS2 infection process at two different steps. When the drug is added prior to infection, viral RNA penetration is suppressed; however, this effect is easily avoided by synchronizing the infection in chloramphenicol before adding Miracil. The exact nature of the second effect, which inhibits a function required between 0 and 20 min of infection, remains unclear. Although viral RNA synthesis is reduced in the presence of Miracil, neither double- nor single-stranded RNA synthesis is specifically affected, and the viral RNA species which are synthesized are the same as those produced in the absence of the drug. Especially notable is the absence of a large peak of ribonuclease-resistant material at 6 to 10*S*, such as is found when MS2-infected cells are treated with actinomycin D (9).

In Miracil-treated infected cultures (cf. Fig. 5), there is a small peak of ribonuclease-resistant RNA at 4 to 6*S*. A portion of this is probably attributable to the partial ribonuclease resistance of soluble RNA (16); however, the number of ribonuclease-resistant counts at 4*S* in the infected culture is increased several-fold over the number in the uninfected culture; a small amount of 6*S* material might be present in the leading

edge of the 4S peak. Samples taken as late as 60 min after infection show no increase in the proportion of this small ribonuclease-resistant RNA.

Since Miracil can bind slightly to RNA (20), it may inhibit some step in the MS2 infection process by binding to single- or double-stranded viral RNA. According to the time course of inhibition, the drug interferes with an early stage of infection and apparently does not affect the messenger activity of the RNA or maturation and assembly of the phage particles. Small amounts of drug might be associated with the viral RNA species and prevent either replicase binding or activity, thereby disrupting RNA replication. The fact that even very high concentrations of Miracil have no effect on triphosphate incorporation when the replicase is assayed in vitro argues against this possibility. However, since a cell lysate was the source of enzyme and template, we can conclude only that Miracil does not interact directly with either enzyme or template to inhibit replicase activity; we cannot conclude whether it interferes with enzyme binding or initiation.

Miracil appears to have a marked effect on the activity of the viral RNA synthetase produced in its presence (Fig. 6). Since these enzyme assays were also performed on whole cell lysates, several variables (e.g., decreased endogenous template concentration, variations in nuclease activity, etc.) might be responsible for the drop in enzyme activity after 20 min in Miracil. It seems improbable that the loss in activity at later times of infection results from the binding of drug to template. If this were the case, inhibition should also be observed at earlier times, when the ratio of drug to viral RNA species should be much higher. Although these data are not inconsistent with the possibility that Miracil somehow alters the viral RNA synthetase, much more detailed studies on the enzyme synthesized in the drug's presence would be required before it could be cited as the target of Miracil action.

It is interesting to note that addition of two other drugs which inhibit bacterial RNA synthesis—actinomycin D and rifampin—during the first few minutes after infection with RNA phage also inhibits the progeny phage yield, presumably because some host component or components required for infection are not being synthesized in their presence (1, 7, 9). Prime candidates for such components would be host factors similar to those which are required for normal activity of the Qβ RNA synthetase (6). The mode of action of Miracil might be similar to that of the other two drugs. Indeed, if host factors required for the viral replicase were not synthesized in sufficient quantities or were abnormal in the presence of Miracil, this would explain why the enzyme which is made is unstable.

Despite the fact that Miracil has an inhibitory effect on the MS2 infection process, it is a useful tool for suppression of host RNA and protein synthesis during studies of RNA phage replication (3). Virus-specific functions apparently are unaffected when the drug is added later than 20 min postinfection. Addition before this time reduces the amount of phage-specific RNA synthesis and inhibits progeny phage production, but these effects are reversible, and the virus-specific species which are made—almost certainly the RNA and probably the protein—do not differ significantly from those made in the absence of the drug.

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